IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application No.: 10/669,824	)
In re application of: JIANG, Cai-Zhong	)
Filed: 23 September 2003	)
Art Unit: 1638	)
Examiner: KRUSE, David H.	)
Docket No. MBI-0034CIP	)
Customer No. 47334	)

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 CFR 1.132 OF OLIVER RATCLIFFE

I, Oliver J. Ratcliffe, declare:

1. I received my Bachelor of Arts degree in Genetics from Cambridge University and my doctoral degree in Biological Sciences from the John Innes Centre at the University of East Anglia, Norwich, United Kingdom. I joined Mendel Biotechnology in June 1999 and served as Director of Research from January of 2004 and then as Vice-President of Research since December of 2005. In this declaration, I serve as an expert witness in that my work has involved the isolation and characterization of plant genes and the use of cloned genes to modify a variety of traits in genetically transformed plants, specifically in the areas of plant developmental biology, the genetic control of flowering, and regulation of environmental stress responses in plants. I have directed research in the area of water deficit tolerance of plants overexpressing sequences of the present invention, and I am therefore familiar with the present invention. This declaration is being drafted as part of my normal duties to support research and intellectual property at Mendel Biotechnology, Inc. As compensation for employment at Mendel Biotechnology, I receive salary, benefits and stock options.

2. This application relates to compositions and methods for modifying a plant's traits. The compositions include plants comprising polynucleotides that encode novel plant transcription factor polypeptides first identified in *Arabidopsis thaliana*, a plant used experimentally as a model plant species. The methods include using the polynucleotides and their encoded polypeptides to modify a trait in a transgenic plant, such as the tolerance of a plant to water deficit.

3. For the purposes of this declaration, a plant "line" means the progeny (through seed or vegetative propagation) of a transformation event or a newly bred variety (specific genotype).

4. I understand that the Examiner rejected claims in the present application as failing to comply with the written description and enablement requirements of 35 U.S.C. §112, first paragraph, and the novel and non-obviousness requirements of 35 U.S.C. §102 and of 35 U.S.C. §103.

5. Applicants identified closely related homologs by pair-wise BLAST analysis by aligning a set of reference sequences against a set of test sequences. Applicants then performed a phylogenetic analysis of the related sequences. See, for example, similar methods described by Thompson et al. (1994) *Nucleic Acids Res*. 22: 4673-4680. The phylogenetic analysis was also similar to that performed on page 34, lines 3-10 of the specification and by Eisen (Eisen (1998) *Genome Res*. 8: 163-167). Once a phylogenic tree for a gene family of one species was constructed using a program such as CLUSTAL, potential orthologous sequences could be placed into the phylogenetic tree and their relationship to genes from the species of interest can be determined. Thus, once closely-related sequences were identified, their function could be inferred by determining the function of the reference sequence (Eisen, *supra*). Applicants thus identified numerous sequences using BLAST and phylogenetic analysis known in the art. These sequences are presented in Exhibit B.

Using methods found in Example VIII of the specification beginning on page 87 at line 17, or in Exhibit A, Applicants then tested the inference in the specification that sequences homologous to those listed in the Sequence Listing, for example, to sequences closely-related to G3456, SEQ ID NOs. 13 and 14, identified by BLAST analysis (page 94, line 33 to page 95, line 2 of the specification) and phylogenetic analysis (page 34, lines 3-10 of the specification), generally perform similar biological functions to one another. These functions include, for example, increasing tolerance to a water deficit stress, which can be measured with assays for tolerance to salt, sucrose, heat, or drought. The results conducted to date with a limited number of transformed plant lines confirm this inference and are noted below.

6. I herewith submit experimental results obtained by overexpressing a number of sequences closely related to the G3456 DNA sequence (SEQ ID NO: 13, which encodes SEQ ID NO: 14) found by the method described in the specification using the BLASTp program with default parameters of the BLOSUM62 scoring matrix (as described in the specification on page 71, line 29-page 72, line 19). These sequences were also subjected to BLAST and phylogenetic analysis and found to be closely related to G3456 by virtue of having descended from a common ancestral sequence. The sequences listed in Table 1, below, were each overexpressed in *Arabidopsis thaliana* using transformation methods described in Examples I- IV beginning at page 77 of the specification. These closely-related polypeptide sequences have conserved domains with at least about 73% identity to the

conserved domain of G3456, SEQ ID NO: 92). To date, 5 of 6 of these closely-related sequences overexpressed in plants have been shown to confer increased tolerance to a water deficit-related stress, including salt, heat, sucrose, desiccation, and/or drought. The sixth sequence has not yet been fully tested, as noted below.

Table 1. Increased water deficit tolerance including salt, heat, sucrose, or drought tolerance conferred by G3456 and closely-related homologs under the regulatory control of the 35S promoter

GID	Plant species from which sequence is derived	Percent identity of second conserved domain to second conserved domain of G3456, SEQ ID NO: 92	Increased salt, heat, sucrose, and/or drought tolerance observed
G3456	*G. max*	100% (96/96 identical residues)	+[1]
G3460	*G. max*	75.0% (72/96 identical residues)	+[2]
G3459	*G. max*	73.9% (71/96 identical residues)	+[3]
G2153	*A. thaliana*	74.0% (77/104 identical residues)	+[4]
G3401	*O. sativa*	75% (72/96 identical residues)	+[5]
G3457	*G. max*	75% (72/96 identical residues)	-[6]

\+ At least three lines (except where indicated*) of plants ectopically expressing the GID in column 1 were more tolerant to a water-deficit-related stress in a plate-based assay than control plants, and/or at least two lines showed a higher rate of survival than controls following drought treatment in soil-based assays.

[1] Transformed plant lines were more tolerant to 150 mM salt than controls and more tolerant to drought in soil-based assays.

[2] Transformed plant lines were more tolerant to 32° C than controls and more tolerant to drought in soil-based assays. *Two lines were more tolerant to 9.4% sucrose than controls.

[3] Transformed plant lines were more tolerant to 150 mM salt or to 32° C than controls. Soil-based drought assays have not yet been performed.

[4] Transformed plant lines were more tolerant to 150 mM salt, to 9.4% sucrose than controls, and were more tolerant to drought in soil-based assays.

[5] Transformed plant lines were more tolerant to 9.4% sucrose than controls and more tolerant to drought in soil-based assays. *Two lines were also more tolerant to 150 mM salt than controls.

[6] Transformed plant lines showed a comparable performance to controls in a limited set of plate based abiotic stress assays conducted thus far, but these lines have not yet been tested in soil-based drought assays.

7. In light of the facts that:

(a) Applicants disclosed that G3456, SEQ ID NOs: 13 and 14, "may be also used to alter a plant's response to water deficit conditions and, therefore, could be used to engineer plants with enhanced tolerance to drought, salt stress" (page 95, lines 11-12 of the specification);

(b) Applicants correctly predicted at, for example, page 39, lines 18-31, or page 102, lines 31-33 of the specification that sequences orthologous to those provided in the Sequence Listing derived from *Arabidopsis thaliana* or from other plants may function similarly in plants, including by increasing abiotic stress tolerance, including osmotic stresses such as drought or salt stress;

(c) Applicants disclosed that the close relationship of two nucleic acid molecules may be determined by percentage identity and alignment of conserved domains, or by using a hybridization-based approach (for example, in the section beginning at page 40, or in Example IX, beginning at page 95, line 15), and that a domain may be a portion of a polypeptide which performs at least one biological function of the intact polypeptide in substantially the same manner or to a similar extent as does the intact polypeptide (page 58, lines 24-29); and

(d) Applicants have shown that at least five of the six sequences homologous to SEQ ID NOs: 1 and 2 with second conserved domains having at least about 73% identity to the similar domain of G3456, SEQ ID NO: 14, that have been overexpressed in plants may be used to confer increased water deficit tolerance;

it is my contention that the specification described the claimed invention in such a way and in sufficient and appropriate detail that, at the time the application was filed, the inventor(s) were in possession of the claimed invention, and that other skilled artisans could routinely practice the invention and readily identify sequences and plants encompassed by the claims.

8. I hereby declare that all statements made herein are true and that they are based on my own knowledge, information and belief. These statements are made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issued from it.

Date: 7 January 2008

Oliver J Ratcliffe

Oliver J. Ratcliffe, Ph.D.
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Exhibit A. Assay Methods

Experiments were performed with the *Arabidopsis thaliana* ecotype Columbia (col-0). Expression of a given transcription factor (TF) from a particular promoter was generally achieved using either a direct-promoter fusion construct in which that TF was cloned directly behind the promoter of interest or by a two component system. For experiments in which G2153 was overexpressed in plants, the direct fusion system and two-component system were used. For the latter, two separate constructs were used, each carrying the different components: Promoter::LexA-GAL4TA and opLexA::TF. The first of these (Promoter::LexA-GAL4TA) comprised a desired promoter cloned in front of a LexA DNA binding domain fused to a GAL4 activation domain, whereas the second comprised the TF of interest cloned behind a DNA target (operator) site for the LexA DNA binding domain. A transgenic line homozygous for the Promoter::LexA-GAL4TA construct was first established and this was then supertransformed with the second construct containing the opLexA::TF fusion and a different selectable marker. In the experiments conducted with the sequences reported in this declaration, each polynucleotide sequence related to G3456 was overexpressed under the regulatory control of the cauliflower mosaic virus 35S (CaMV 35S, or 35S) promoter.

<u>Plate-based assays</u>

Plate-based physiological assays representing a variety of stress related conditions were used to identify stress-tolerant lines. All plate assays were performed under sterile conditions. Plants were grown and assayed under controlled temperature and humidity on sterile medium so as to minimize variability in the results obtained. All assays were designed to detect plants that were more tolerant to the particular stress condition and were developed with reference to the following publications: Jang et al. (1997) *Plant Cell* 9: 5-19, Smeekens (1998) Cu*rr. Opin. Plant Biol.* 1: 230-234, Liu and Zhu (1997) *Proc. Natl. Acad. Sci. USA* 94: 14960-14964, Saleki et al. (1993) *Plant Physiol.* 101: 839-845, Xu et al. (1996) *Plant Physiol.* 110: 249-257, Zhu et al. (1998) *Plant Cell* 10: 1181-1191, Alia et al. (1998) *Plant J.* 16: 155-161, Xin and Browse (1998) *Proc. Natl. Acad. Sci. USA* 95: 7799-7804, and Leon-Kloosterziel et al. (1996) *Plant Physiol.* 110: 233-240. Where possible, assay conditions were originally tested in a blind experiment with controls that had phenotypes related to the condition tested.

Prior to plating, seed for all experiments were surface sterilized in the following manner: (1) 5 minute incubation with mixing in 70 % ethanol, (2) 20 minute incubation with mixing in 30%

bleach, 0.01% triton-X 100, (3) 5X rinses with sterile water, (4) Seeds were re-suspended in 0.1% sterile agarose and stratified at 4 °C for 3-4 days.

Assays were generally performed on non-selected segregating T2 populations in order to avoid the extra stress of selection. Control plants for assays on lines containing direct promoter-fusion constructs were wild type or Col-0 plants transformed with an empty transformation vector (pMEN65). Controls for 2-component lines (generated by supertransformation) were wild type or the background promoter-driver lines (i.e. promoter::LexA-GAL4TA lines), into which the supertransformations were initially performed.

<u>Germination assays</u>

All germination assays followed modifications of the same basic protocol. Sterile seeds were sown on the conditional media that had a basal composition of 80% MS + Vitamins. Control seeds were incubated at 22 °C under 24-hour light (120-130 $\mu E\ m^{-2}\ s^{-1}$) in a growth chamber. Evaluation of germination and seedling vigor was performed 5 days after planting.

Heat germination assays were conducted as above, but the plants were germinated at 32° C.

Salt stress germination assays were conducted by sowing sterile seeds of controls and transgenic plants overexpressing the G3456-related polynucleotides on conditional media that had a basal composition of 80% MS + Vitamins + 150 mM NaCl (the concentration disclosed in the present specification). The plates were incubated at 22° C under 24-hour light (120-130 $\mu E\ m^{-2}\ s^{-1}$) in a growth chamber. Evaluation of germination and seedling vigor was performed five days after planting.

Tolerance to sucrose was determined in media and with incubation periods similar to that described for salt stress germination assays, except that the overexpressors and control plants were grown on a basal composition of 80% MS + Vitamins + supplemented with 9.4% sucrose rather than 150 mM NaCl .

<u>Growth assays</u>

For heat sensitivity (32 °C) growth assays, seeds were germinated and grown for 7 days on MS + vitamins + 1% sucrose at 22 °C and then were transferred to heat stress conditions. Heat stress (32 °C) was applied for 5 days, after which the plants were transferred back to 22 °C for recovery and evaluated after a further 5 days.

After each of these stresses and recovery periods, the plants were visually examined and qualitatively scored as more tolerant of the stress if they showed fewer stress symptoms than the controls (such as chlorosis or the production of protective pigments such as anthocyanins).

Soil Drought Assays

The soil drought assay (performed in clay pots) was based on that described by Haake et al. (2002) *Plant Physiol.* 130: 639-648.

Seeds were sterilized by a 2 minute ethanol treatment followed by 20 minutes in 30% bleach / 0.01% Tween and five washes in distilled water. Seeds were sown to MS agar in 0.1% agarose and stratified for 3 days at 4° C, before transfer to growth cabinets with a temperature of 22° C. After 7 days of growth on selection plates, seedlings were transplanted to 3.5 inch diameter clay pots containing 80g of a 50:50 mix of vermiculite:perlite topped with 80 g of ProMix. Typically, each pot contained 14 seedlings, and plants of the transgenic line being tested were in separate pots to the wild-type controls. Pots containing the transgenic line versus control pots were interspersed in the growth room, maintained under 24-hour light conditions (18-23° C, and 90-100 $\mu E\ m^{-2}\ s^{-1}$) and watered for a period of 14 days. Water was then withheld and pots were placed on absorbent diaper paper for a period of 8-10 days to apply a drought treatment. After this period, a visual qualitative "drought score" from 0-6 was assigned to record the extent of visible drought stress symptoms. A score of "6" corresponded to no visible symptoms whereas a score of "0" corresponded to extreme wilting and the leaves having a "crispy" texture. At the end of the drought period, pots were re-watered and scored after 5-6 days; the number of surviving plants in each pot was counted, and the proportion of the total plants in the pot that survived was calculated.

Analysis of results. In a given experiment, we typically compared six or more pots of a transgenic line with six or more pots of the appropriate control. The mean drought score and mean proportion of plants surviving (survival rate) were calculated for both the transgenic line and the wild-type pots. In each case a *p*-value was calculated, which indicated the significance of the difference between the two mean values.

Calculation of p-values . Survival was analyzed with a logistic regression to account for the fact that the random variable is a proportion between 0 and 1. The reported *p*-value was the significance of the experimental proportion contrasted to the control, based upon regressing the logit-transformed data.

Drought score, being an ordered factor with no real numeric meaning, was analyzed with a non-parametric test between the experimental and control groups. The *p*-value was calculated with a MannWhitney rank-sum test.